RESEARCH COMMUNICATION

Putative capacitative calcium entry channels: expression of *Drosophila trp* and evidence for the existence of vertebrate homologues

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Capacitative calcium entry is a major pathway through which intracellular calcium stores are refilled after stimulation. It has been suggested that the protein encoded by the transient receptor potential (*trp*) gene expressed in *Drosophila* photoreceptors may be homologous with capacitative calcium entry channels. Expression of the *trp* gene product in *Xenopus* oocytes led to significant increases in calcium entry only when the intracellular calcium stores were depleted. Previous investigations have found *trp* to be uniquely expressed in *Drosophila* photoreceptors, but PCR cloning shows that homologous proteins exist in *Calliphora*, mouse brain and *Xenopus* oocytes. It is thus possible that capacitative calcium entry in *Xenopus* oocytes is mediated by a homologue of *trp*.

INTRODUCTION

Agonist stimulation of many non-excitable cells evokes a biphasic cytoplasmic calcium signal, with an initial transient rise in cytosolic calcium concentration due to release of calcium from inositol 1,4,5-trisphosphate-sensitive stores and a secondary sustained rise which is entirely dependent on calcium influx across the plasma membrane [1]. This calcium-influx pathway appears to be activated by depletion of intracellular calcium stores, irrespective of the manner in which this is achieved, and has been termed capacitative calcium entry [2–5]. Electrophysiological characterization of this pathway reveals a depletion-activated low-conductance channel highly selective for calcium [6,7]. However, very little is known about the molecular components of the capacitative calcium-influx pathway.

It has been suggested recently that proteins homologous with the *Drosophila* photoreceptor transient receptor potential (*trp*) calcium channel may be responsible for capacitative calcium influx [1,5,8,9]. The light response in *Drosophila* photoreceptors is known to occur downstream of phospholipase C activation and the production of inositol 1,4,5-trisphosphate [8]. Electrophysiological recordings comparing the light-evoked currents in wild-type and in *trp*-mutant *Drosophila* photoreceptor cells have shown that the *trp*-dependent current is highly selective for calcium [8]. The fact that *trp* is activated downstream of inositol 1,4,5-trisphosphate production and is a calcium-selective channel has led to the suggestion that the *trp* protein may be homologous with the capacitative-calcium-entry channel.

Recently, the *Drosophila trp* protein has been expressed in the insect cell line Sf9, where it was shown that a novel calcium-selective current was induced that could be activated by depletion of intracellular calcium stores [9]. A related protein also found in *Drosophila* photoreceptors, the *trp*-like (*trpl*) protein, has also been expressed in Sf9 cells and has been shown to form a non-selective cation channel permeable to calcium which is not activated by store depletion [9,10], but is regulated by the cytosolic inositol 1,4,5-trisphosphate concentration [11,12].

From the data from *Drosophila* photoreceptors and the expression studies in insect cells, it thus appears that both *trp* and *trpl* are involved in gating calcium fluxes across the insect cell plasma membrane downstream from inositol 1,4,5-trisphosphate production. In this study we show that the expression of the *trp* protein in a vertebrate cell (the *Xenopus* oocyte) significantly increases capacitative calcium entry, suggesting that *trp* may function as a capacitative calcium entry channel. Although *trp* and *trpl* have previously been suggested to be *Drosophila* photoreceptor-specific proteins [13–16], PCR cloning shows that proteins homologous with the *Drosophila trp* protein are found in *Calliphora*, mouse brain and *Xenopus* oocytes. We thus demonstrate for the first time the existence of a mammalian homologue of *trp* and, importantly, that a homologue of *trp* exists in *Xenopus* oocytes, in which the capacitative calcium entry pathway has been well studied [17–20]. Proteins homologous with *trp* are thus candidates for capacitative calcium entry channels.

MATERIALS AND METHODS

Electrophysiological measurements

Changes in cytosolic calcium concentration were monitored by electrophysiological recordings of the calcium-activated chloride current, as described previously [19]. Briefly, oocytes were impaled in the animal pole by two microelectrodes (2 M KCl-filled, resistance 0.5–2 MΩ) and voltage was clamped to −60 mV using a Warner Instruments oocyte clamp amplifier (OC-725B). All experiments were carried out at room temperature, 18–22 °C. The perfusates contained (mM): 115 NaCl, 2 KCl, 1 $MgCl_2$, 5 Hepes (pH 7.4 adjusted by NaOH) with either 2 mM $CaCl_2$ added to give a calcium-containing perfusate or 1 mM EGTA added to give a calcium-free perfusate. To deplete the intracellular calcium stores, the oocytes were incubated in calcium-free medium with 1 mM EGTA and 1 μM thapsigargin (0.1% DMSO) for over 3 h, which has previously been shown to activate capacitative calcium entry and to empty completely the intracellular stores [19].

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The nucleic acid sequence data reported in this paper appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X90696 (*Xenopus*), X90697 (mouse) and X90698 (*Calliphora*).

RNA expression

The *trp* cDNA, *ctrp*-9, in pBluescript KS (a gift from C. Montell) was linearized by using the restriction endonuclease *Kpn*I, and cRNA was synthesized with the mCAP mRNA capping kit (Stratagene) by using T7 polymerase, following the manufacturer's protocol. After injection of *trp* cRNA, oocytes were maintained for more than 24 h at 17 °C to allow for protein translation. Control oocytes were injected with an equal volume of buffer. Calcium entry was monitored by electrophysiological recordings.

PCR cloning

cDNA was prepared by random-primed reverse transcription of RNA isolated from *Drosophila* heads, *Calliphora* heads, mouse brain and *Xenopus* oocytes. Degenerate oligonucleotide primers were designed by using the *Drosophila trp* [13,14] and *trpl* [15] nucleotide sequences. The 5′-primer was ATIAA$^{C}_{T}$CCICA$^{C}_{T}$ $^{C}_{T}$TIGGICC (bases 1773–1792 of *trp* sequence [13]) and the 3′-primer was TC$^{C}_{T}$TC$^{A}_{G}$AA$^{A}_{G}$TAI$^{C}_{G}$ $^{A}_{T}$-CATCCA (bases 2251–2270 of *trp* sequence [13]). The PCR reaction mixture contained cDNA, 20 nmol of dNTPs, 100 pmol of each primer, and 2.5 units of Taq polymerase (Boehringer Mannheim) in the supplied 1.5 mM $MgCl_2$ buffer. Reactions were carried out for 30 cycles each of 1 min at 94 °C to denature, 1 min at 50 °C for annealing and 1 min at 72 °C for extension. PCR products obtained from *Drosophila*, *Calliphora* and mouse brain were fractionated on a 1 % agarose gel, purified with the Wizard PCR kit (Promega) and cloned into the plasmid vector pGEM-T (Promega). Plasmids containing inserts of the correct size were sequenced from both strands by using Sequenase V2.0 (USB) and automated PCR-based fluorescence sequencing (ABI 373A). Consensus sequences from three independent clones of each tissue (*Drosophila*, *Calliphora* and mouse brain) were obtained to eliminate errors introduced by the PCR amplification. These sequences were aligned and new primers were designed based on conserved regions: 5′-primer TT$^{C}_{T}$G$^{C}_{G}$I$^{A}_{T}$ $^{A}_{G}$ $^{C}_{T}$GGI$^{C}_{T}$ TIAA$^{C}_{T}$CA (bases 1879–1898 of *trp* sequence [13] and 3′-primer $^{C}_{G}$ $^{A}_{T}$IC$^{G}_{T}$IGC$^{A}_{G}$AA$^{C}_{T}$TTCCA$^{C}_{T}$TC (bases 2224–2243 of *trp* sequence [13]. These primers were used to amplify a *trp* homologue from *Xenopus* oocytes as described above.

RESULTS AND DISCUSSION

Assessment of capacitative calcium entry

Application of 2 mM calcium pulses to *Xenopus* oocytes otherwise perfused with calcium-free medium with 1 mM EGTA evoked no changes in current under voltage-clamp conditions ($n = 7$; Figure 1a). This indicates that under normal resting conditions when the intracellular calcium stores are full, there is no observable calcium entry. However, after a 3 h pretreatment with 1 μM thapsigargin (a specific endoplasmic reticulum calcium-ATPase inhibitor), large calcium entry-evoked currents can be observed with great reliability in oocytes when 2 mM calcium pulses are applied ($n = 58$; Figure 1b). This shows that a capacitative calcium entry pathway is present in *Xenopus* oocytes, in agreement with previous studies [17–20]. The capacitative calcium entry pathway in *Xenopus* oocytes can be blocked reversibly by application of 1 mM Zn^{2+} ($n = 5$; Figure 1c), as shown previously [19], and this provides a useful tool for probing the calcium entry channels. The amplitude of the calcium entry evoked currents recorded from thapsigargin-pretreated oocytes can thus be used to quantify calcium entry via the capacitative pathway, and the specificity of the evoked currents

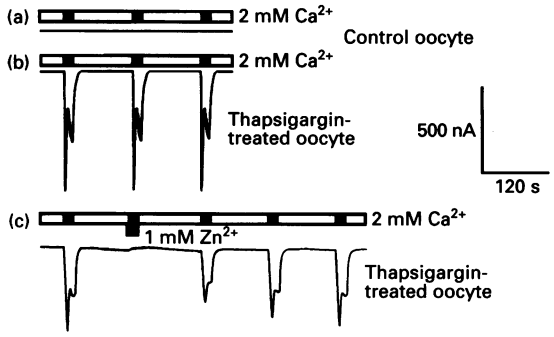

Figure 1 Assessment of capacitative calcium entry

(**a**) Calcium pulses applied to a control *Xenopus* oocyte with full intracellular calcium stores evoked no detectable calcium entry. (**b**) Thapsigargin-pretreated oocytes showed large calcium entry evoked currents, indicating the presence of a capacitative calcium entry pathway. (**c**) The thapsigargin-evoked calcium entry could be completely and reversibly blocked by application of 1 mM Zn^{2+}.

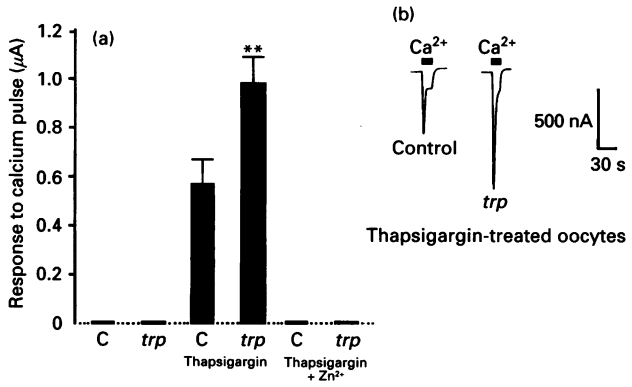

Figure 2 Expression of the calcium channel encoded by *trp* in *Xenopus* oocytes potentiates capacitative calcium entry

(**a**) *Xenopus* oocytes expressing the *trp* protein did not show calcium entry-evoked currents when the intracellular calcium stores were full, but when the stores were depleted by thapsigargin, calcium entry-evoked currents were significantly potentiated (**, $P < 0.02$) in oocytes expressing *trp* compared with control oocytes. The calcium entry-evoked currents after thapsigargin treatment could be blocked by application of 1 mM Zn^{2+} in both *trp* cRNA-injected and control (C) oocytes. (**b**) Expression of the *trp* protein in *Xenopus* oocytes increased calcium entry-evoked currents, when the intracellular calcium stores are depleted by thapsigargin pretreatment.

can be checked by application of zinc. We have utilized these methods to investigate the effects on capacitative calcium entry of expressing the *Drosophila trp* protein in *Xenopus* oocytes.

The protein encoded by *trp* may be a capacitative calcium entry channel

The *Drosophila trp* protein was expressed in *Xenopus* oocytes by injecting approx. 10 ng of cRNA made by transcription *in vitro* of the cDNA (a gift from C. Montell) and allowing 24 h for translation. Immunoblots revealed the existence of an approx. 150 kDa protein recognized by the *αzctrp* antiserum (a gift from C. Montell) in cRNA-injected oocytes, but not in control oocytes (results not shown). No calcium current is active in the control or the *trp*-injected oocytes (Figure 2a) when the calcium stores

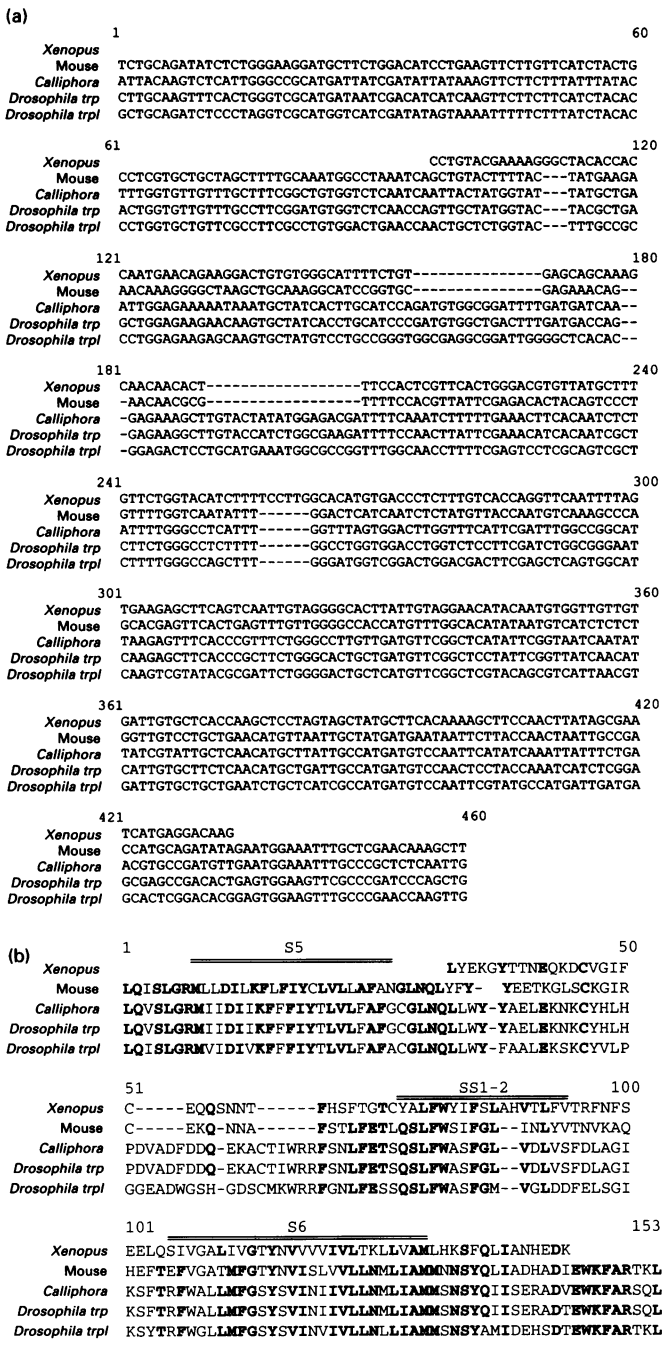

Figure 3 PCR cloning indicates the presence of proteins homologous with *trp* in *Calliphora*, mouse brain and *Xenopus* oocytes

(**a**) Nucleotide sequences of PCR products from *Drosophila, Calliphora* and mouse brain were obtained by using primers immediately adjacent to the sequences shown. The *Drosophila* sequences found were identical with the published sequences for *trp* [13,14] and *trpl* [15]. The *Calliphora* sequence is 80% identical with the *trp* sequence, whereas the mouse brain sequence is 62% identical. Further PCR using new primers to the most conserved regions of these sequences revealed a *Xenopus* oocyte homologue, having 53% identity with the mouse brain sequence. (**b**) Predicted amino acid sequences of the PCR products shown in (**a**). The *Calliphora* and mouse brain sequences show 99% and 56% identity with the *trp* sequence respectively. The *Xenopus* oocyte homologue shows 46% identity with the mouse brain homologue. Amino acids which are identical in four or more of the sequences are shown in bold.

are full. Cells expressing *trp* show 72% (highly significant, $P < 0.02$) larger calcium entry-evoked currents (0.98 ± 0.11 μA; mean $\pm$ S.E.M.; $n = 15$) after thapsigargin pretreatment than do control injected oocytes (0.57 ± 0.10 μA; $n = 15$) from the same batch (Figures 2a and 2b). A separate batch of oocytes injected with 0, 0.8 or 8 ng of *trp* cRNA showed increases in calcium entry-evoked currents after thapsigargin pretreatment above control of 0%, 63 ± 54% and 137 ± 64% ($n = 5$) respectively. The calcium entry-evoked currents can be completely blocked by 1 mM Zn^{2+} in both control ($n = 4$) and *trp*-injected oocytes ($n = 4$) (Figure 2a). Whereas previous results have indicated that the expression of *trp* in an insect cell line gives rise to a depletion-activated calcium current [9], these experiments indicate that *trp* expressed in a vertebrate cell may also function as a capacitative calcium entry channel.

Proteins homologous with *trp* can be found in *Calliphora*, mouse brain and *Xenopus* oocytes

Northern- and Western-blot analysis along with immunolocalization in previous studies have suggested that both *trp* and *trpl* are specifically localized to *Drosophila* photoreceptors [13–16]. Since capacitative calcium entry has been observed in many cell types, any candidate for the capacitative calcium entry channel must have a wide tissue distribution of homologous proteins. In order to determine whether *trp* fulfils this criterion, we probed various tissues, using the PCR technique. PCR primers were designed to amplify the most conserved region between the *trp* and *trpl* cDNAs, which contains the putative fifth and sixth transmembrane regions, as well as a region similar to the pore-lining SS1-2 region of other cation channels. PCR amplification of *Drosophila*, *Calliphora* and mouse brain cDNA revealed bands of the expected size. Sequencing of the PCR products showed that the *Drosophila* band corresponded to the known *trp* and *trpl* sequences. Homologous sequences were found in *Calliphora* (80% identity at the nucleotide level, and 99% at the amino acid level) and mouse brain (62% identity at the nucleotide level and 56% at the amino acid level)(Figures 3a and 3b). Based on consensus sequences between these peptide sequences, new PCR primers were designed and tested on *Xenopus* oocytes. Sequencing of the PCR products from *Xenopus* oocytes revealed the presence of a cDNA with 53% identity at the nucleotide level and 46% identity at the amino acid level with the mouse brain *trp* homologue (Figures 3a and 3b). The *Calliphora*, mouse brain and *Xenopus* oocyte sequences do not show homology with any published sequences other than *trp* [13,14] and *trpl* [15]. The mouse brain clone indicates for the first time that a mammalian homologue of *trp* exists. The *Xenopus* oocyte *trp* homologue shows that a cell known to have capacitative calcium entry expresses a *trp* homologue.

Drosophila trp thus appears to be activated by calcium-store depletion not only in insect cells [9] but also in a vertebrate cell (Figure 2). *Drosophila trpl* is not stimulated by the emptying of intracellular stores, but is activated by inositol 1,4,5-trisphosphate [11,12]. Both *Drosophila trp* and *trpl* are thus intimately involved in gating calcium across the plasma membrane in the phosphoinositide signalling cascade, and the vertebrate homologues (Figure 3) may fulfil a similar role. The vertebrate homologues of *trp*/*trpl* are thus potential candidates for the capacitative calcium entry channels. Since *Xenopus* oocytes do not appear to express any plasma-membrane calcium channels other than those involved in capacitative entry [19], it is possible that the *Xenopus* oocyte homologue of *trp* may form the capacitative calcium entry channel in this tissue.

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